

Effects of several flavonoids on the growth of B16F10 and SK-MEL-1 melanoma cell lines: relationship between structure and activity

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Although flavonoids seem to be capable of acting at all stages of the carcinogenic process, little information is available on their action in melanoma cell lines. The aim of this study was to assess the response of B16F10 and SK-MEL-1 melanoma cell lines to treatment with six different flavonoids after 24 and 72 h of exposure and to relate the response to their structure. We then compared the findings with those for melphalan treatment. When cultures were treated for 24 h, only slight inhibition at the highest concentrations (25 and 50 μM) of tangeretin and luteolin were observed, whereas melphalan caused a dose-related inhibition of growth at all concentrations. Quercetin, hesperetin, 7,3'-dimethylhesperetin and eriodictyol did not produce any effect at 24 h on B16F10 or SK-MEL-1 cells, results which point to the low toxicity of flavonoids. After 72 h of exposure culture growth was inhibited by 7,3'-dimethylhesperetin at 50 μM , but lower concentrations had no effect. Tangeretin was the most effective of the flavonoids in inhibiting B16F10 and SK-MEL-1 cell growth, showing a clear dose-response curve after 72 h. These results suggest that the absence of the C₂-C₃ double bond on hydroxylated flavonoids results in a loss of effect on both the cell lines, while the higher activity of tangeretin compared with 7,3'-dimethylhesperetin suggests that the presence of at least three adjacent methoxyl groups confers a more potent antiproliferative effect. © 2002 Lippincott Williams & Wilkins

Key words: Antiproliferative, B16F10, 7,3'-dimethylhesperetin, eriodictyol, flavonoids, hesperetin, *in vitro*, luteolin, melanoma, melphalan, quercetin, SK-MEL-1, tangeretin, treatment

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Introduction

Plant flavonoids have attracted much recent attention as important dietary cancer chemoprotective agents.¹⁻³ The possible antitumour action of certain flavonoids has also generated interest.⁴⁻⁶ Flavonoids seem to be capable of acting at all stages of the carcinogenic process, including damage to the DNA, tumour growth and invasion. Some flavonoids have been shown to have an *in vitro* antiproliferative effect on various neoplastic cell lines, for example myeloid and lymphoid leukaemia cells,⁷ gastric cancer cells,⁸ ovarian cancer cells,⁹ squamous cell carcinoma,⁴ prostate cancer cells,¹⁰ colon adenocarcinoma cells,¹¹ breast cancer adenocarcinoma and human neuroblastoma cells,¹² and thyroid cancer cells.¹³ Flavonoids may affect the enzymes involved in signal transduction (kinases, phospholipases and phosphodiesterases)¹⁴⁻¹⁶ and may regulate other enzymes critical for cell growth. Several flavonoids are thought to have oestrogenic agonist/antagonist activity.¹⁷ Other mechanisms proposed include the inhibition of tyrosine protein kinase,^{18,19} DNA topoisomerases^{20,21} and ribosomal S6 kinase activity²² or phosphatidylinositol breakdown.²³ These studies seem to indicate that the inhibition of different key enzymes after receptor binding could play an important role in the growth inhibition of malignant cells.

Melanoma is the eighth most commonly diagnosed disease in the USA,²⁴ and its incidence is rising world-wide at a rate of about 5% per year.²⁵ Chemo-

therapy of late diagnosed and metastatic melanoma cases with dacarbazine and alkylating agents has important side effects. Moreover, melanoma has one of the worst rates of response to chemotherapy of all neoplasias.²⁶ These problems have led to the search for new types of treatment²⁷ and for new compounds with fewer side effects.

Flavonoids seem to fit these requirements. However, few studies have been carried out in melanoma, and most of those that have been performed have been concerned with the metastatic process, such as the study in which several polyphenolic compounds were tested for their ability to inhibit lung metastasis induced by B16F10 melanoma cells in mice.²⁸ New findings show that apigenin and quercetin inhibit melanoma (B16-BL6) lung colonization.²⁹

In the present study, the *in vitro* effects of several plant flavanones, flavones and flavonols on the growth and proliferation of a mouse (B16F10) and human melanoma (SK-MEL-1) cell line were investigated, and the antiproliferative activity was compared with that of the drug melphalan. The possible relationships between the antiproliferative properties of these flavonoids and their structure and biological activities are discussed.

Materials and methods

Cell cultures

The B16F10 cell line (established from a primary cutaneous mouse C57BL/6 melanoma) was kindly provided by Dr Hearing from the National Cancer Institute (Bethesda, Maryland, USA). The SK-MEL-1 cell line originated from a lymphatic metastasis of a human cutaneous melanoma and was established as a cell line at the Memorial Sloan-Kettering Cancer Center. It was obtained from the American Type Culture Collection (ATCC, Maryland, USA).

The *Mycoplasma* species test was carried out to confirm its absence throughout the study. The B16F10 and SK-MEL-1 lines were cultured in Eagle's minimum essential medium (EMEM), supplemented at 10% with fetal bovine serum (FBS) and streptomycin plus penicillin (100 µg/ml and 100 u/ml, respectively). Non-essential amino acids and 1 mM of sodium pyruvate were added when culturing SK-MEL-1.

All the processes were carried out in a Cultair ASB type II vertical laminar flow chamber. The B16F10 and SK-MEL-1 cultures were kept at 37°C, 98% relative humidity and 5% CO₂ atmosphere in a

Cytoperm heater. The culture medium was changed every 2 days or when acidification was indicated by the pH indicator (phenol red).

Chemicals and reagents

Hesperetin, eriodictyol, luteolin and quercetin were obtained from Furfural Español S.A. (Murcia, Spain). 7,3'-Dimethylhesperetin was synthesized by Furfural Español S.A. according to the patent number PE 9902037. Tangeretin was obtained from Extrasynthèse (Genay, France) (Figure 1). Melphalan, sodium pyruvate, streptomycin, penicillin, phosphate buffered saline (PBS), bovine serum albumin (BSA) fraction V and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were obtained from Sigma Co. (Madrid, Spain). Ethanol and dimethyl sulphoxide (DMSO) (both high performance liquid chromatography reagent grade) were obtained from Merck (Darmstadt, Germany). EMEM, and amino acids were purchased from GIBCO (USA).

Preparation of chemicals for administration in cell cultures

Melphalan was first dissolved with ethanol acidified with HCl (to improve stability) and then mixed with phosphate buffer to obtain the stock concentrations (3 mM). All the steps were performed in flow chambers and melphalan was kept on ice during its preparation to prevent inactivation. The vials were then stored at -80°C. In order to determine the maximal stable concentration of flavonoids that can be achieved *in vitro*, DMSO stock was diluted in PBS, PBS with BSA (20 mg/ml) and ultrapure water (18.2 M Ω, MilliQ-UF-Plus, Millipore, USA). Different dilutions of each compound ranging from 1:5 to 1:500 were centrifuged at 13,000 g (Microfuge-K, Beckman) for at least 20 s. The concentrations at which a stable solution formed were ascertained from observing the vials with no pellet formation. We selected PBS, PBS with BSA or water for each flavonoid on the basis of the maximal stable concentration obtained.

Cell viability quantification: MTT test

The methods of Carmichael *et al.*^{30,31} and Alley *et al.*³² were adapted to our culture conditions. Briefly, the cultures were incubated in 96-well plates with 200 µl of fresh supplemented medium and 50 µl of

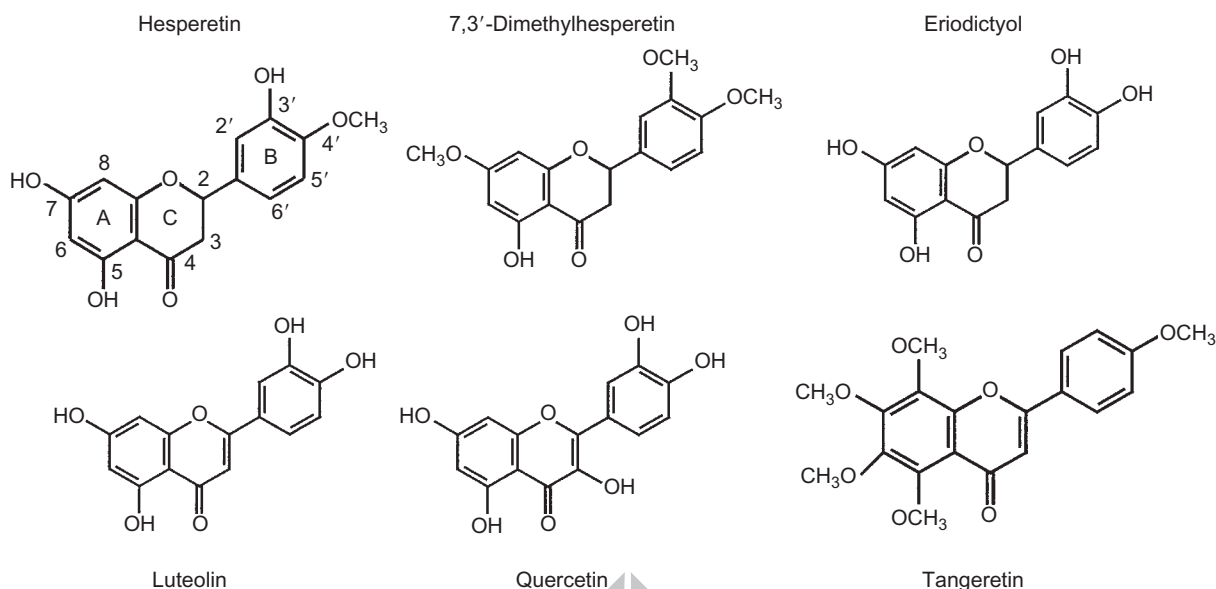


Figure 1. The chemical structures of the six flavonoids used: hesperetin, 7,3'-dimethylhesperetin, eriodictyol, luteolin, quercetin and tangeretin.

MTT (8 mg/ml) for 4 h at 37°C and 5% CO₂. After centrifugation (240 g for 10 min) to carefully remove the medium and non-metabolized MTT, 100 µl of DMSO were added to each well to solubilize the MTT formazan produced by the cultured cells. After shaking for 30 min at room temperature, the plates were read with a Multiskan MCC/340P spectrophotometer, using 570 nm for the reading and 690 nm for the reference wavelength.

Treatments

We carried out two types of *in vitro* assays to evaluate the cytotoxicity and antiproliferative capability of several flavonoids. In this study the following cytotoxicity and antiproliferative concepts were used. Effects seen after treatment for 24 h must be related to cytotoxicity since growth inhibition in short-time experiments will be caused by direct cell death (this mechanism is well known for melphalan) or apoptosis induction, whereas in long-time exposure assays (72 h) quiescence or metabolic stopping can also occur in addition to growth inhibition by cytotoxicity, and therefore antiproliferative action is a more accurate concept. To evaluate cytotoxicity, B16F10 and SK-MEL-1 cells were obtained from exponential non-confluent cultures and seeded at 2500 cells/well in 96-well plates. They were kept in fresh supplemented medium for 24 h to allow cell

adhesion and environmental adaptation. They were then treated with hesperetin, 7,3'-dimethylhesperetin, eriodictyol, luteolin, quercetin or tangeretin for 24 h. Melphalan, a known cytotoxic melanoma drug,²⁷ was used for comparison. After 24 h of treatment, the medium was replaced and the cultures were maintained for another 24 h before the MTT assay was carried out.

To evaluate the possible antiproliferative effects of flavonoids, 100 and 2500 cells/well of B16F10 and SK-MEL-1, respectively, were seeded in 96-well plates. After 24 h to allow for culture adaptation, melphalan, hesperetin, 7,3'-dimethylhesperetin, eriodictyol, luteolin, quercetin or tangeretin were added and incubated in normal culture conditions for 72 h. The plates were then centrifuged (240 g for 10 min), the medium was replaced and the cultures maintained for another 24 h before the MTT assay was carried out.

Data analysis

All the experiments were performed at least in triplicate and the results were recorded graphically. In the graphs, each point corresponds to a flavonoid concentration and is the mean of four to six measurements. The bars that appear in some graphs are calculated from the standard error of the mean. Paired Student's t-tests were carried out to compare

the percentages of surviving cells in the cultures at different concentrations of the various compounds.

Results

Cell growth experiments: flavonoid cytotoxicity

When B16F10 and SK-MEL-1 cultures were treated with the six selected flavonoids for 24 h we were unable to observe any dose-response curve for quercetin, hesperetin, 7,3'-dimethylhesperetin or eriodictyol. Only tangeretin and, to a lesser extent, luteolin at their highest concentrations (25 and 50 μM) produced slight inhibition, which was more obvious in B16F10 than in SK-MEL-1 (Figures 2 and 3). With melphalan, on the other hand, a clear dose-response dependency was obtained when cultures were treated in the same conditions. Melphalan-treated B16F10 cultures showed significant differences from flavonoid-treated B16F10 cultures at all concentrations ($P < 0.05$). In the case of melphalan-treated SK-MEL-1 cultures, significant differences ($P < 0.05$) from the flavonoid treatments were observed at melphalan concentrations greater than 10 μM . As can be seen in Figures 2 and 3, B16F10

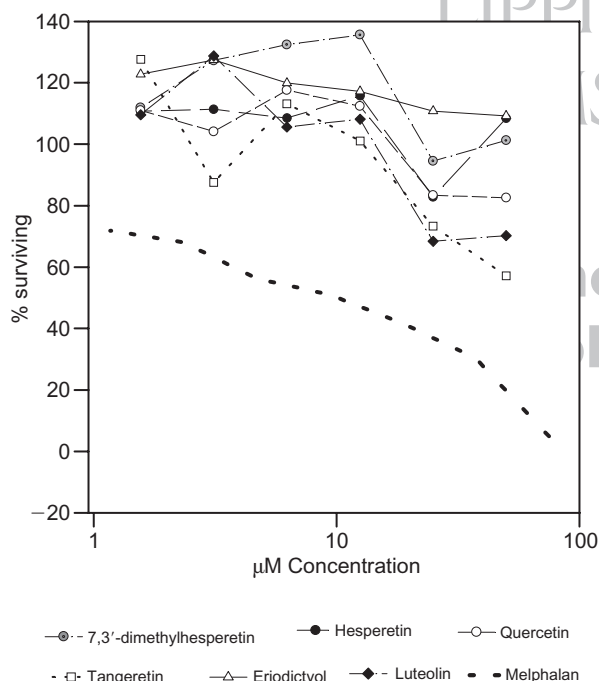


Figure 2. Cell viability of B16F10 after 24 h of treatment with melphalan, hesperetin, 7,3'-dimethylhesperetin, eriodictyol, luteolin, quercetin or tangeretin. Each point is the mean of four to six measurements.

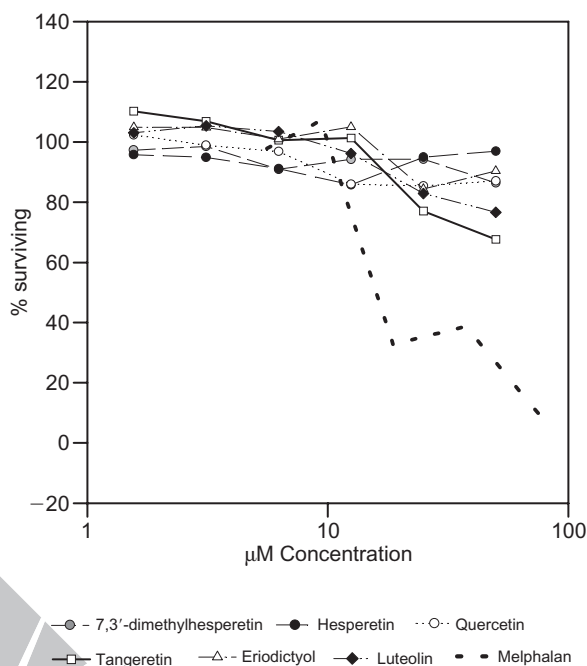


Figure 3. Cell viability of SK-MEL-1 after 24 h of treatment with melphalan, hesperetin, 7,3'-dimethylhesperetin, eriodictyol, luteolin, quercetin or tangeretin. Each point is the mean of four to six measurements.

cultures showed greater sensitivity than SK-MEL-1 cultures for the 24 h treatments, although the more variable response to the treatments of B16F10 compared with SK-MEL-1 cultures should also be noted.

Cell growth experiments: antiproliferative effects of flavonoids

The inhibition of B16F10 and SK-MEL-1 cell growth caused by the flavonoids studied was determined according to the data from MTT reduction assays after exposure to the flavonoids for 72 h. The effects of different concentrations of these flavonoids on the growth of both melanoma cell lines are illustrated in Figures 4 and 5 (B16F10) and 6 and 7 (SK-MEL-1).

The 72 h treatments applied to the B16F10 line (Figure 4) showed no dose-response curve for hesperetin or eriodictyol. Only the other flavanone 7,3'-dimethylhesperetin at the highest possible dose (50 μM ; above this dose spontaneous insolubilization in the culture medium leads to the formation of crystals visible on phase contrast microscopy) caused significantly ($P < 0.005$) lower growth with respect to the control (40% of cell viability expressed as a percentage of control cells). Quercetin

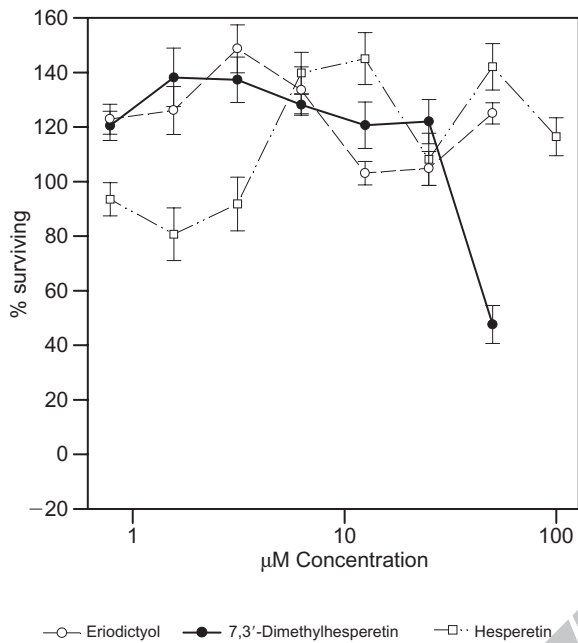


Figure 4. Cell viability of B16F10 after 72 h of treatment with hesperetin, 7,3'-dimethylhesperetin or eriodictyol. Each point is the mean of four to six measurements. The bars are calculated from the standard error of the mean.

and luteolin treatment of B16F10 produced a dose-response curve that represented inhibition at the highest concentration of 33.4% and 21.5%, respectively (Figure 5). The 72 h tangeretin treatment was the best at inhibiting growth, with a clear dose-

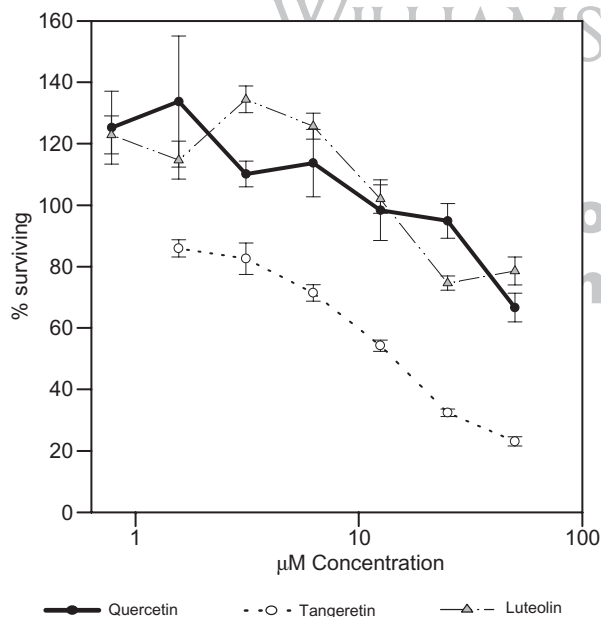


Figure 5. Cell viability of B16F10 after 72 h of treatment with luteolin, quercetin or tangeretin. Each point is the mean of four to six measurements. The bars are calculated from the standard error of the mean.

response curve being obtained that represented inhibition ranging from 14.2% to 77% and an ID₅₀ of about 10 μM. Moreover, the results at all tangeretin doses showed significant differences from those obtained with quercetin and luteolin ($P < 0.01$) (Figure 5).

When SK-MEL-1 cultures were treated for 72 h with hesperetin and eriodictyol no growth inhibition was observed (non-significant differences were observed when the highest and lowest doses were compared) and, as in the case of B16F10, only the 50 μM concentration of 7,3'-dimethylhesperetin caused growth inhibition (30.3%) with respect to the controls ($P < 0.01$) (Figure 6). Exposure of SK-MEL-1 cultures to quercetin (25 μM) and luteolin (50 μM) led to inhibition between of 20% to 10%, whereas no inhibition was observed at the lower doses (Figure 7). Tangeretin treatment of SK-MEL-1 produced inhibition of growth that was proportional to the dose, being 32.2% at 50 μM, with significant differences between the 50, 25 and 12.5 μM treatments ($P < 0.01$) (Figure 7).

When B16F10 and SK-MEL-1 cultures were exposed to melphalan for 72 h, clear dose-response curves were observed, with the former cell line showing greater sensitivity and significant differences at 7.5, 3.75, 1.8 and 0.9 μM ($P < 0.01$) and an ID₅₀ of 1 μM and 7.5 μM, respectively, for the two types of cells (Figure 8). Compared with the most

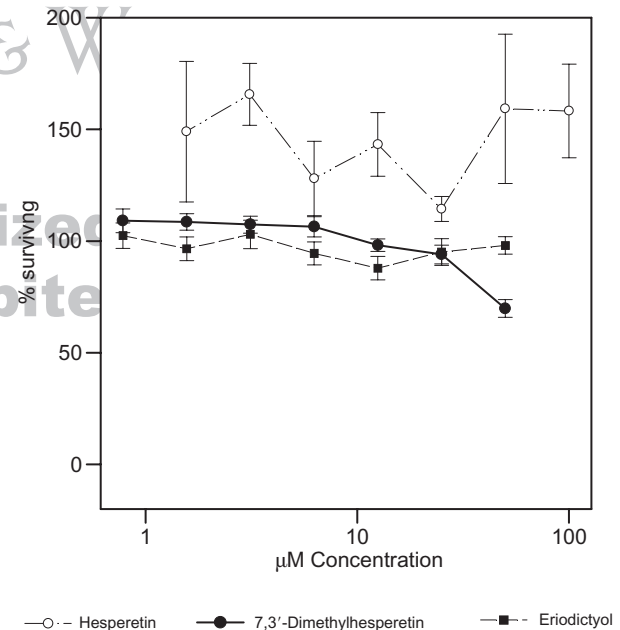


Figure 6. Cell viability of SK-MEL-1 after 72 h of treatment with hesperetin, 7,3'-dimethylhesperetin or eriodictyol. Each point is the mean of four to six measurements. The bars are calculated from the standard error of the mean.

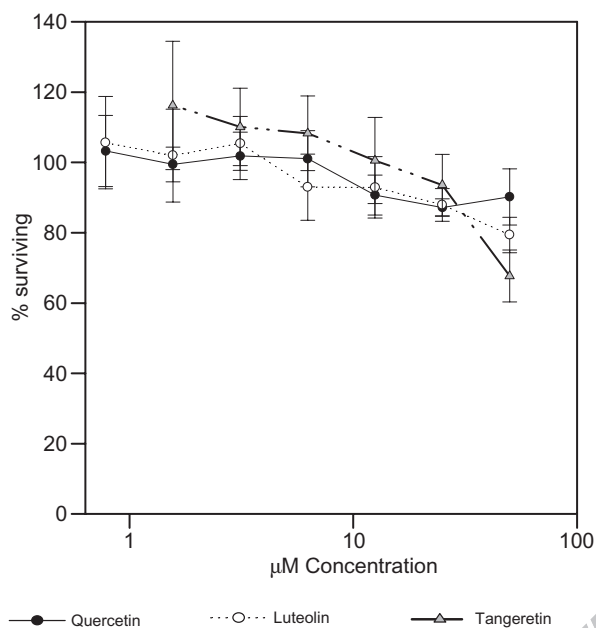


Figure 7. Cell viability of SK-MEL-1 after 72 h of treatment with luteolin, quercetin or tangeretin. Each point is the mean of four to six measurements. The bars are calculated from the standard error of the mean.

effective flavonoid (tangeretin), which also produced a dose-response curve for both cell lines, the ID_{50} for melphalan was 10 times lower. The results showed that, in all cases but particularly with melphalan and tangeretin, B16F10 was more sensitive and responsive to drugs than SK-MEL-1, both at 24 h (Figures 2 and 3) and 72 h (Figure 8).

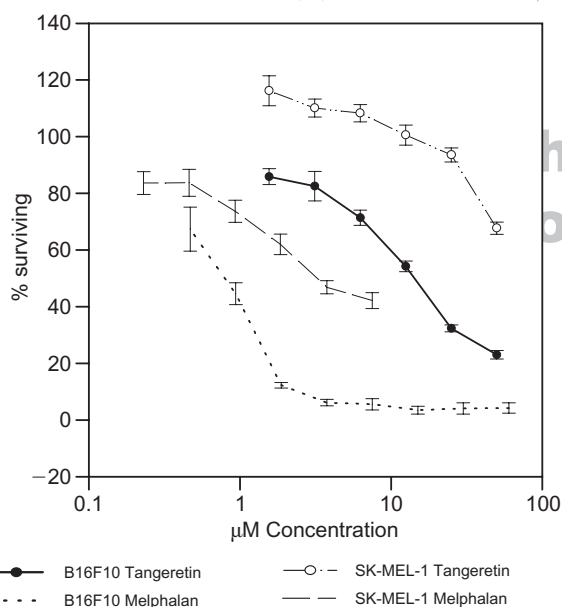


Figure 8. Cell viability of B16F10 and SK-MEL-1 after 72 h of treatment with melphalan or tangeretin.

Discussion

According to the data from the MTT reduction assays shown in Figures 4, 5, 6, 7 and 8, the flavonoids tangeretin and 7,3'-dimethylhesperetin significantly inhibit B16F10 and SK-MEL-1 cultures (the latter flavonoid only being effective at its highest achievable concentration); luteolin and quercetin only had a moderate effect on the growth of the B16F10 cell cultures (Figure 5). The MTT reduction assay also pointed to the lack of effect of the flavanones hesperetin and eriodictyol on both cell growth and viability at all the concentrations used. The percentage of viable cells did not differ from those observed in control conditions (Figures 4 and 6).

Figure 5 suggests that the effect of tangeretin is not only due to the cytostatic activity of the flavone, since the inhibition of B16F10 growth increased in a dose-dependent way. Figure 4 shows that the reduction in cell numbers observed at 50 µM of 7,3'-dimethylhesperetin may be related to a cytotoxic effect, since such inhibition was not dose-dependent and occurred only at the highest concentration used. Further studies are necessary to clarify the behaviour of this flavanone.

The results showed that the B16F10 line was more sensitive to treatments than the SK-MEL-1 line, both with melphalan and flavonoids, which reflects our previous observation²⁷ that the higher resistance of SK-MEL-1 and lower resistance of B16F10 are characteristics of each cell line; these attributes may be related to their respective origins (mouse primary tumour culture and human metastatic melanoma).

In most of the published studies concerning the antineoplastic activities of flavonoids cell lines such as thyroid,¹³ breast,³³⁻³⁷ cervix,³⁷ non-small cell lung carcinoma³⁸ and cholangiocellular carcinoma³⁹ were used. This seems to indicate a general preference for using cell lines originated from glandular or oestrogen target tissue. Briefly, with these lines, flavonoids such as luteolin, apigenin,¹³ citrus flavonoids,³⁴ 7-methoxyflavanone and 7,8-dihydroxyflavone,³⁵ kaempferol, genistein and quercetin³⁸ showed growth inhibition potential at concentrations ranging from 0.1 µM to 20–30 µM. No such inhibition was observed when our melanoma cultures were treated with luteolin or hesperetin.

However, few studies have been carried out with melanoma cultures.⁴⁰ Quercetin treatment for 6 days produced marked inhibition (45%) but only at the highest dose used (10 µM) when added daily to the medium (60 µM final concentration), whereas 3 days' treatment did not produced any inhibition,⁴⁰ results that seem to be in accordance with ours for

quercetin and 7,3'-dimethylhesperetin. In other reports using quercetin stronger responses were obtained (IC₅₀ values of 15 and 65 µM, respectively) with non-melanoma cell lines (HT29¹¹ and HuCC-T1³⁹) than with the SK-MEL-28 melanoma cell line⁴⁰ or the B16F10 and SK-MEL-1 lines used in our study. Surprisingly, we have been unable to find any reports on the *in vitro* treatment of melanoma with tangeretin, which was the most effective of the six flavonoids tested in inhibiting the *in vitro* growth of both melanoma lines used; the IC₅₀ values obtained were comparable, at least for B16F10, to results obtained with other flavonoids in thyroid,¹³ breast³³⁻³⁸ and lung carcinomas.^{39,40}

Depending on their structure, flavonoids display more or less powerful inhibitory effects on the growth and proliferation of certain malignant cells *in vitro*. Previous studies suggest that the position, number and substitution of the hydroxyl groups of the A and B rings and saturation of the C₂-C₃ bond may be important factors affecting flavonoid cytotoxic and/or antiproliferative activities. Thus, myricetin (3',4',5'-trihydroxyflavone) was a more powerful phosphatidylinositol 3-kinase α inhibitor than luteolin or apigenin.⁴¹ Similarly, the inhibitory activities of baicalin and wogonin at low doses were lower than that of baicalein, indicating that the presence of three hydroxyl groups in the 5, 6 and 7 positions (A ring) seems to be necessary for inhibitory activity towards the proliferative response of smooth muscle cells.⁴² Flavones and flavonols were the most powerful flavonoids affecting the cell viability of colonic adenocarcinoma HT29 cells, whereas flavan-3-ols, flavanones and isoflavones were inactive.¹¹ The antiproliferative effects of different flavonoids on several tumour cultures (MCF7 human breast cancer, and SHEP and WAC2 human neuroblastoma-derived cells) show that a non-hydroxylated C ring with oxofunction at position 4 and a C₂-C₃ double bond is required for maximal biological activity.¹² All these data suggest that the effects of flavonoids on malignant cell growth differ, with results and patterns being specific for each cell line. Even the presence of certain radicals in the flavonoid skeleton seems to modify the antimitotic activity in an unpredictable manner.

With regard to these flavonoid structure-activity relationships, our results clearly show that only the polymethoxylated or *O*-dimethoxylated flavonoids (flavones and flavanones) significantly affected cell viability in the two models used, whereas the *O*-dihydroxylated flavones and flavonols displayed only a weak effect and the mono- and *O*-dihydroxylated flavanones were inactive.

These results suggest that the absence of the C₂-C₃ double bond in hydroxylated flavonoids results in loss of their inhibitory activity on both melanoma cell lines used. Since the effect of luteolin is either similar to (B16F10) or more pronounced than (SK-MEL-1) that of quercetin, it seems also that 3-hydroxylation in the flavone nucleus does not confer a more powerful inhibitory effect. However, the principal structural components that can affect these antiproliferative properties are the number and the position of methoxyl groups in the A and B rings of the flavone and flavanone skeleton. The higher activity of tangeretin compared with 7,3'-dimethylhesperetin in the B16F10 cell line (also observed in SK-MEL-1, but not so pronounced) suggests that the presence of at least three adjacent methoxyl groups confers a more potent antiproliferative effect.

In summary, the order of potency of flavonoids used on cell growth inhibition is tangeretin > 7,3'-dimethylhesperetin > luteolin = quercetin for the B16F10 cell line, and tangeretin = 7,3'-dimethylhesperetin > luteolin > quercetin for the SK-MEL-1 cell line.

Flavonoids have attracted attention as potentially important dietary cancer chemoprotective agents on the basis of their biological activities at the cellular level, and various hypotheses have been proposed to explain their anticancerous properties.¹⁴ Our results using melanoma cell lines clearly show that there is no correlation between the antiproliferative effects of the flavonoids used and their antioxidant and/or free radical scavenging activities. The most potent antioxidant group in the flavonoid nucleus, the B ring catechol structure, is insufficient to inhibit cell proliferation, and the presence of several methoxyl groups in the flavonoid skeleton significantly reduces the antioxidant and free radical scavenging activities of flavonoids.^{3,43} Therefore, further studies are necessary to elucidate the cellular mechanism underlying the antiproliferative effects of some flavonoids in melanoma cell lines.

Acknowledgements

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